Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3139–3142, August 1974

Partial Purification of Human Interferon by Affinity Chromatography

(antiviral glycoprotein/immunoabsorbance)

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Contributed by C. B. Anfinsen, May 20, 1974

ABSTRACT **Human interferon prepared by challenge of leukocytes with Sendai virus, or of fibroblasts with double-stranded poly(inosinic acid)·poly(cytidylic acid), has been studied with respect to purification by affinity chromatography. Both leukocyte and fibroblast interferons are removed from crude tissue culture fluids by means of columns of antibody to leukocyte interferon attached to Sepharose-4B. The antibody was prepared in sheep using, as antigen, material that had been partially purified by gel filtration through Sephadex G-100 columns. Many of the impurities in the crude fibroblast interferon were presumably not recognized by the sheep antibodies induced by leukocyte interferon. Fibroblast interferon was, therefore, much more effectively purified as the result of this "common denominator" approach. The fibroblast product, in contrast to interferon from leukocytes, could only be harvested efficiently from the crude starting material when a carrier protein (bovine-serum albumin, and later, cytochrome c) was added to the eluting buffers to counteract losses, presumably due to adsorption on purification and assay equipment. Both varieties of interferon exhibit molecular weights of approximately 20,000–25,000, although association with higher molecular weight proteins occurs.**

Studies on a number of species including man have shown that interferon possesses genus- or family-specific antiviral activity against a broad spectrum of viruses. Most of the relevant literature suggests that interferon is a glycoprotein having a molecular weight of approximately 25,000 (1, 2).

This potentially valuable antiviral agent shows an extremely high specific activity and it has been estimated that 1 mg of pure interferon may be equivalent to as much as 10^9 international units (a unit causes 50% reduction of viral multiplication in a standard tissue culture system). The crude batches of leukocyte material supplied to us for use in the experiments described below generally contain approximately 5×10^6–10^7 units in 400 ml. Even if isolation methods avoided significant losses, enormous volumes would be required to produce amounts of the agent suitable for definitive clinical trials in man. For this reason we have undertaken the isolation of human interferon in sufficient quantity for structure determination and possible eventual synthesis. This paper on human interferon outlines an immunoabsorbant-affinity chromatography procedure, a technique which was shown previously to be of value in the purification of mouse interferon (3, 4), and reports our findings on some basic physical and chemical properties, many of which have been investigated in the past on interferons from other species.

MATERIALS AND METHODS

Human interferon, prepared by K. Cantell and Smith, Kline and French Laboratories, was supplied to us by the Antiviral Substances Program of the National Institute for Allergy and Infectious Diseases. It was prepared by challenge of human leukocytes with Sendai virus as described by Cantell and his colleagues (5), and various samples contained between 12,000 and 25,000 international units/ml. Assays, by a modification of the micro method originally described for rabbit kidney cells (6), were carried out under contract with the North American Biological Association, Inc., Rockville, Md., and reproducibility was controlled by the inclusion of coded samples of known dilutions of a frozen stock solution. In this assay the titer of human reference interferon, no. 6919, is 5000 units/ml.

A second variety of human interferon was prepared by Dr. J. Vilcek and his colleagues at New York University, using the FS-4 strain of human foreskin diploid fibroblasts. This interferon was induced with a double-stranded complex of poly(inosinic acid)·poly(cytidylic acid), and high yields were obtained by use of pretreatment of cultures with cycloheximide and actinomycin (7). These cultures also yielded interferon at a level of approximately 20,000 units/ml before concentration.

As others have observed (8, 9) in studies on several species, interferon shows remarkable stability to low pH. We have, therefore, effected partial purification by gel filtration on columns of G-100 Sephadex-Fine (Pharmacia Fine Chemicals, Inc.) using, as solvent, 0.1 M acetic acid, 0.15 M NaCl, pH 3.0. Columns were run at room temperature and fractions were collected in siliconized glass test tubes or plastic test tubes. Before application to columns, crude interferon was either thoroughly dialyzed at 4° against 0.1 M acetic acid and lyophilized, or concentrated, before dialysis against 0.1 M acetic acid–0.15 M NaCl, to a concentration of approximately 25–30 mg/ml by Amicon pressure filtration using a 65-mm UM10 filter disc (Amicon Ultrafiltration Cell, model 202). Essentially no losses of interferon activity occurred during concentration by filtration or dialysis and lyophilization.

Affinity columns were prepared by means of the CNBr activation method described by Axén and Porath (10) as applied to affinity chromatography in general, and to the preparation of immunoabsorbant columns (11–13). The affinity chromatography principle was used not only for concentration and purification of interferon as described below in *Results*, but also for the removal of antibodies in the crude γ-globulin fraction of serum from sheep that had received partially purified leukocyte interferon. Antiserum to the partially purified leukocyte interferon was produced in a sheep which, after initial immunization, was given 8 bi-weekly injections. Each injection consisted of approximately 1 mg of the active fraction, having about 10^6 units of interferon

Abbreviation: PBS, phosphate-buffered saline.

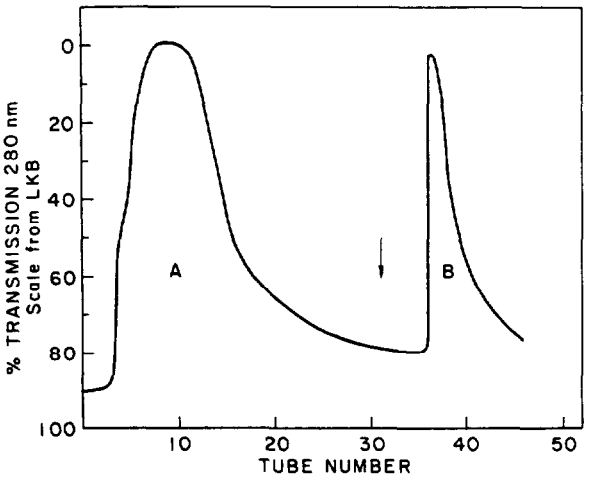

FIG. 1. Removal of antibodies against proteins known to be present in crude leukocyte interferon. A solution (ml·A_{280} = 2220) in PBS of the γ-globulin fraction of the serum from sheep no. 991 was applied to the "impurities"-agarose column described in *Materials and Methods*. Unadsorbed protein was washed through with PBS and bound antibodies were then eluted with 0.1 M citrate buffer, pH 2.2 (*arrow*). Additional elution with 8 M urea in PBS caused the release of only a small additional amount of protein. γ-globulin in peak *A*, total ml·A_{280} = 1576; material in peak *B*, total ml·A_{280} = 202.5.

activity, from a G-100 Sephadex column (see *Results*). The antigen, in 1 ml of isotonic saline, was emulsified with an equal volume of complete Freund's adjuvant. Serum from the final bleeding of this sheep was fractionated with ammonium sulfate to yield the γ-globulin components. Thus, in a typical preparation, 825 ml of saturated ammonium sulfate (reagent grade) was slowly added to 1375 ml of serum. The pH was adjusted to 7.4 with 2 N NaOH and the mixture was slowly stirred for 30 min at room temperature. The precipitate was then centrifuged off and redissolved in phosphate-buffered saline (PBS). This volume was adjusted to 1375 ml with PBS and the γ-globulins were precipitated again by adding 688 ml of saturated ammonium sulfate. The precipitate was centrifuged off as before. This step was repeated once again. The final precipitate was dissolved in PBS and dialyzed against

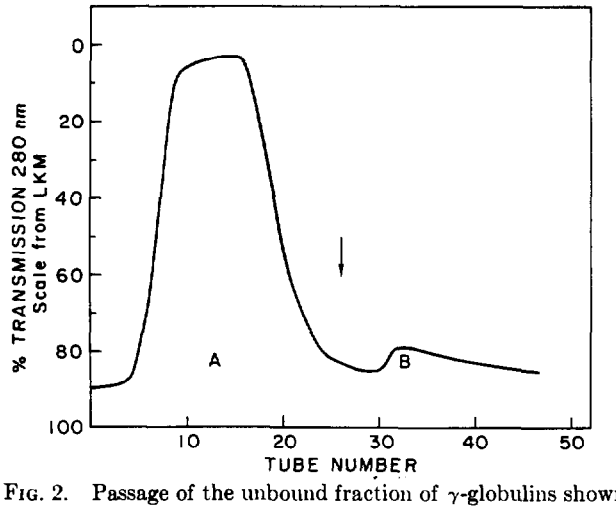

FIG. 2. Passage of the unbound fraction of γ-globulins shown in Fig. 1 through the "impurities"-agarose column after thorough equilibration with PBS. Peak *A*, total ml·A_{280} = 1206; peak *B*, total ml·A_{280} = 29.9.

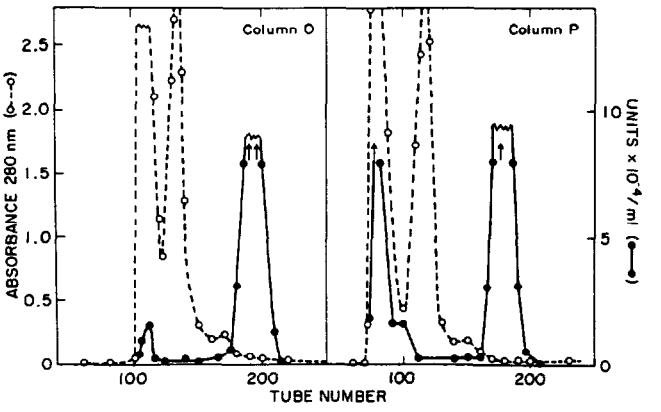

FIG. 3. Gel filtration of the proteins present in crude leukocyte interferon through a column (3.5 × 200 cm) of G-100 Sephadex in 0.1 M acetic acid–0.15 M NaCl. After concentration by ultrafiltration, the proteins (total ml·A_{280} = 1504 and 1350, respectively), were applied to Columns O and P, respectively. Recoveries of interferon activity in the major peaks were approximately 20% of the activity applied. The low recovery of activity may be related to the length of time required to develop the columns (about 4 days at room temperature). Furthermore, the protein concentration in the major active fractions was quite low, a factor known to contribute to losses during purification and assay.

6000 ml of cold PBS, with three changes. The final solution (1010 ml) had an optical density of 0.67 after 1:100 dilution with PBS.

Antibodies against those proteins thought to constitute the major impurities in the antigen used to immunize the sheep were removed by passing the γ-globulin solution through an "impurities"-agarose column. To prepare a typical column of this type, 100 mg of material derived from buffy coat cells by passage through a French press with subsequent dialysis against 0.1 M acetic acid and lyophilization, 350 mg of serum proteins (similarly dialyzed and lyophilized), and 10 mg of mixed allantoic fluid–Sendai virus proteins were taken up to 20 ml of PBS and coupled to 30 ml of CNBr activated agarose (12, 13). We are grateful to Dr. Sheldon Wolff and Dr.

TABLE 1. *Anti-human interferon titer in sheep no. 991*

Serum	Interferon 1/10	1/100	1/1000
1. Normal 1/10	3.1	2.1	0.7
1/100	2.8	1.6	0.5
1/1000	3.0	1.7	0.2
2. After 5th boost 1/10	2.0	<0	<0
1/100	2.8	1.4	<0
1/1000	3.3	1.7	<0
3. After 8th boost 1/10	1.0	0.3	<0
1/100	1.4	<0	<0
1/1000	2.8	<0	<0

Equal volumes of serum and of various dilutions of interferon were mixed and incubated at 37° for 1 hr. The mixtures were assayed (6) for interferon activity and results are expressed as $\log_{10}$ titers of interferon. Dilutions of both serum and the stock interferon solution (12,000 units/ml) were made in Eagle's minimum essential medium with added glutamine and Hepes buffer.

Charles Dinarello for human buffy coat cells and for human serum, and to Drs. H. Levy and S. Baron for hen's egg allantoic fluid containing Sendai virus. Aliquots of approximately 30 ml of the γ-globulin solution were passed through the "impurities"-agarose column; after regeneration of the column with 0.1 M citrate buffer, pH 2.2, and subsequent thorough washing with PBS, the protein that passed through this column, unretarded, on the first run was passed through a second (and occassionally a third) time. In general, very little material was bound upon the second passage of the unretarded PBS fraction through this "scavenger" column. All manipulations were at room temperature except for extended storage of the affinity column at 5° between runs when 0.02% sodium azide was present in the final PBS wash. Figs. 1 and 2 show the elution patterns obtained during two successive runs on the mixed protein column. Later columns, bearing larger amounts of the suspected protein impurities, have yielded anti-interferon globulins which, after only a single pass, are essentially devoid of further adsorbable materials.

RESULTS

Fig. 3 illustrates the results of two typical gel filtration experiments designed to yield leukocyte antigen for immunization. In some such runs, e.g., column P, a fraction of the interferon activity moved through the column with front-running material. Since this front-running activity is eluted (with essentially no loss of activity) from G-100 Sephadex columns run with 6 M guanidine hydrochloride–0.1 M acetic acid at a position consistent with the molecular weight of 20,000–25,000, we suspect that nonspecific adsorption to larger molecules is occurring. Other experiments on gel filtration under a variety of environmental conditions support the idea that suspected oligomeric, or specifically aggregated forms of interferon, may be simply nonspecific aggregates with other larger components of the crude tissue culture fluids.

The immunizations described under *Materials and Methods* employed the pooled protein-containing fractions showing interferon activity, e.g., the active tubes in the region of fraction 180–200 in column P. Neutralization of leukocyte interferon activity by addition of various dilutions of hyperimmune antiserum to various dilutions of stock interferon was used to give a rough measure of the progress of specific anti-interferon production (Table 1). Sheep no. 991 reached a high level of activity after the eighth boost, and its serum (1500 ml) was collected. A second sheep, no. 903, also reached high titer levels and has been retained, without further boosting, as an animal likely to yield a strong anamnestic response when more highly purified interferon becomes available.

The major innovation in the present work has been the use of an immunoabsorbent column prepared with the anti-interferon induced by interferon from one cell type (leukocytes, by the Cantell method) for the purification of interferon derived from another cell type [diploid fibroblasts prepared by the procedures of Vilcek and Havell (7)]. Since many impurities in the leukocyte cultures are likely to be absent from the fibroblast cultures, greater purification might be expected (see also ref. 4). The results shown in Figs. 4 and 5 illustrate that, although more complete binding of leukocyte interferon activity is achieved with the anti-leukocyte interferon column, the amount of protein in the peak eluted with acidic citrate buffer is considerably greater than with the fibroblast material. In a run such as that illustrated in Fig. 5, the PBS fraction that passed through the column unretarded contained 1824 ml·A_{280} units and the fraction eluted with pH 2.2 citrate buffer contained only 12.6 ml·A_{280} units due to protein other than the added serum albumin. Recovery of applied interferon activity units varied between 50% and 100%. We suspect that this variability is due in part to the relatively large error inherent in the biological assay method.

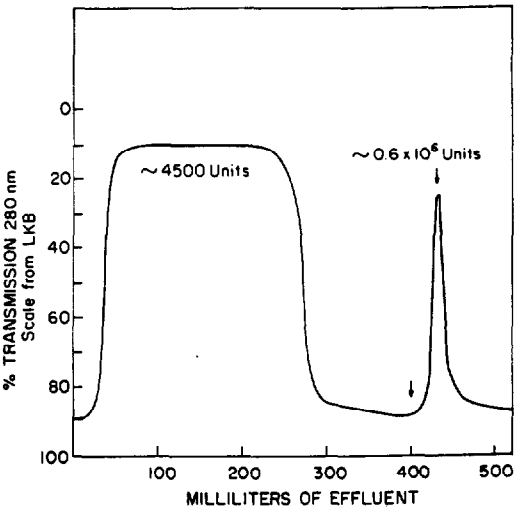

FIG. 4. Purification of human leukocyte interferon on an immunoabsorbant column. The column (2 × 11 cm) was equilibrated with PBS and, after unabsorbed protein in the applied sample of crude interferon (570 mg containing approximately 1.6 × 10^8 units of interferon) had been washed through, bound activity was eluted with citrate buffer, pH 2.2 (*arrow*). The numbers on the ordinate are taken directly from the chart paper supplied by the LKB Co.

Although bovine-serum albumin was used in the experiments illustrated in Fig. 5, it was later found that interferon and albumin occasionally formed a complex that interfered with subsequent purification steps. At present, stabilization and prevention of adsorption to equipment is achieved using horse heart cytochrome *c*. Parenthetically, cytochrome *c* has the added advantage of possessing an *N*-acetyl end group, thus

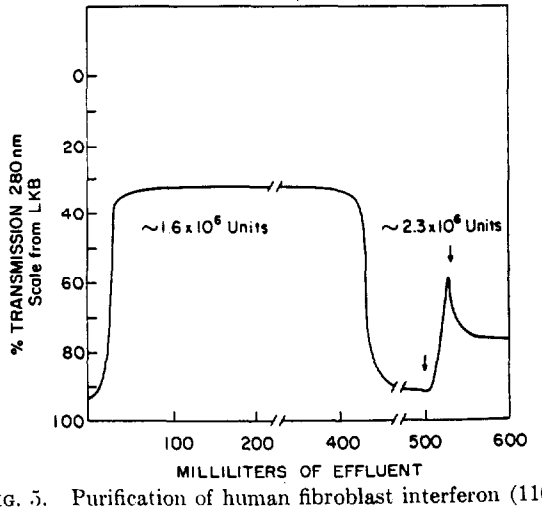

FIG. 5. Purification of human fibroblast interferon (1160 mg with 4 × 10^6 units of activity). The same column and conditions described in the *legend* for Fig. 4 were used in this experiment, except that the citrate buffer at pH 2.2 contained bovine serum albumin (1 mg/ml).

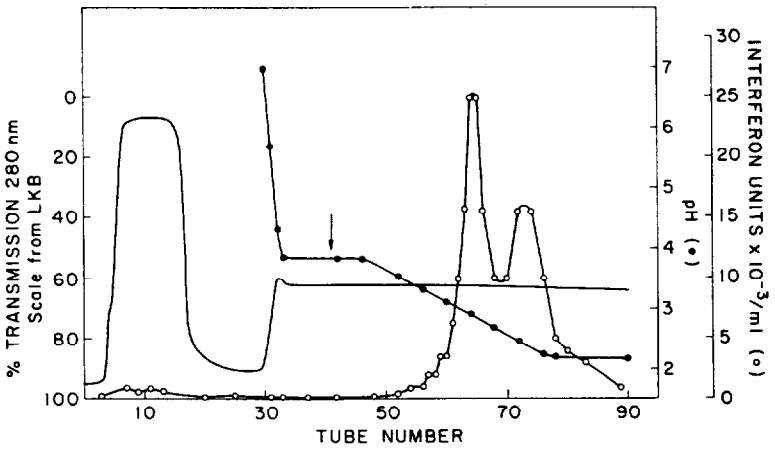

FIG. 6. Purification of human leukocyte interferon (2.3×10^6 units) on an anti-leukocyte interferon column. After loading of the sample, the column was washed with PBS and then with McIlvaine phosphate–citrate buffer pH 3.8, containing 500 μg of cytochrome *c* per ml. Interferon was eluted with a pH gradient, starting as indicated by *arrow*. About 80% of the interferon units were recovered in the two peaks of interferon activity.

being unsusceptible to amino acid sequencing by Edman degradation.

More recently, elution of interferon from the anti-leukocyte interferon column has been carried out by means of a pH gradient. A linear gradient of McIlvaine phosphate–citrate buffer from pH 3.8 to pH 2.2 has been used, with cytochrome *c* in the buffer solutions at a concentration of 500 μg/ml. At the end of the gradient, the column was washed with 0.1 M citrate buffer pH 2.2, before re-equilibration with PBS. As can be seen in Fig. 6, the amount of protein eluted from the column by the pH gradient is less than that observed on elution with citrate buffer pH 2.2 alone in the experiment illustrated in Fig. 4. This is due to the use, in this experiment, of an anti-leukocyte interferon column using anti-interferon γ-globulins that had been passed through an improved "scavenger" column before coupling to Sepharose. The improved "scavenger" column had a larger amount of each of the impurities bound per gram of Sepharose. By means of the gradient elution system, two peaks of interferon were eluted from the anti-interferon column. It therefore appears that there are two species of interferon, differing in their affinity to the anti-interferon γ-globulins. Similar results were obtained on gradient elution of fibroblast interferon from the antileukocyte interferon column. In the case of the fibroblast material, a third species of interferon is present (about 40% of the activity) which is not retarded by the affinity column; the major portion of this activity also escapes binding on a second pass. Whether these differences reflect the presence of a second genetic locus that codes for interferon in fibroblasts, or antigenic differences stemming from incomplete or chemically different embellishment of the polypeptide chain with carbohydrate and/or other non-aminoacid moieties, is not known.

The affinity chromatography method has been of considerable use (unpublished experiments) in the preparation of highly radioactive fibroblast interferon (prepared with the kind cooperation of Drs. J. Vilcek and E. Havell). After subsequent enrichment by gel filtration (as in Fig. 3) and disc gel electrophoresis with the riboflavin catalysis described by Fantes and Furminger (14), such material may be suitable for sequencing studies.

1. Ng, M. H. & Vilcek, J. (1972) *Advan. Protein Chem.* **26**, 173–241.
2. Dorner, F., Scriba, M. & Weil, R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1981–1985.
3. Ogburn, C. A., Berg, K. & Paucker, K. (1973) *J. Immunol.* **111**, 1206–1218.
4. Sipe, J. D., De Maeyer-Guignard, J., Gauconnier, B. & De Maeyer, E. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1037–1040.
5. Strander, H. & Cantell, K. (1966) *Ann. Med. Exp. Biol. Fenn.* **44**, 265–273.
6. Armstrong, J. A. (1971) *Appl. Microbiol.* **21**, 723–725.
7. Vilcek, J. & Havell, E. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3909–3913.
8. Lindenmann, J., Burke, D. & Isaacs, A. (1957) *Brit. J. Exp. Pathol.* **38**, 551–562.
9. Lampson, G. P., Tytell, A. A., Nemes, M. M. & Hilleman, M. R. (1966) *Proc. Soc. Exp. Biol. Med.* **121**, 377–384.
10. Axén, R., Porath, J. & Ernbäck, S. (1967) *Nature* **214**, 1302–1304.
11. Campbell, D. H., Leuscher, E. & Lermann, L. S. (1951) *Proc. Nat. Acad. Sci. USA* **37**, 575–578.
12. Cuatrecasas, P., Wilchek, M. & Anfinsen, C. B. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 636–643.
13. Cuatrecasas, P. & Anfinsen, C. B. (1971) *Annu. Rev. Biochem.* **40**, 259–278.
14. Fantes, K. H. & Furminger, I. G. S. (1967) *Nature* **216**, 71–72.